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Genetic Structure of Natural Populations of Air-breathing Murrel *Channa punctatus* Bloch in the Rohilkhand Plains of India

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Abstract

Using transferrin polymorphs as genetic markers, we have screened the natural population of *Channa punctatus* Bloch that inhabited the Rohilkhand plains of India. Phenotypically, the population was in equilibrium, substantially polymorphic and panmictic with the values of mean heterozygosity (H_{mean}), genetic diversity (H_T), average probability of nonidentity of alleles (H_S), components of gene diversity (D_{ST}) and proportion of gene diversity (G_{ST}) being 0.363, 0.542, 0.292, 0.250 and 0.462, respectively. On the basis of allele frequency data, the entire population was discernible into four distinct groups or subpopulations and each subpopulation occupied a conspicuously demarcated large area or zone. The other distinct features of the population were: an excess of homozygote BB and the nonlethality of any of the alleles with the homozygote AA being rare and appears to be under selection pressure.

Introduction

Out of five species of genus *Channa* distributed in India as well as in several Southeast Asian countries, *C. punctatus* is the most abundant and important food fish. It makes significant contribution to fresh and brackish water capture fishery of these countries, in addition to being stocked as a component of paddy-cum-fish culture (Jhingran 1991). The documentation of its genetic diversity is, therefore, important considering that such information is valuable in breeding programs, genetic stock identification (GSI) and fishery management. Several important points about breeding programs of cultured fish species and their genetics have recently been discussed by Lacy (2000). Fish transferrin (Tf) polymorphs, therefore, make a reliable genetic marker system (Barrett and Tsuyuki 1967; Utter et al. 1970; Valenta et al. 1976;

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Keyvanfar 1986; Van-Doornik and Milner 1996) that has already helped to identify the disappearance of some of the carp races due to prolonged isolation of stocks (Csizmadia et al. 1995). Some evidence also correlates Tf levels of sera with certain pathological conditions of a few fish species, since the bacteria can directly uptake iron bound to transferrins (Winter et al. 1980; Hirono and Aoki 1996).

The two main objectives of our investigations are: 1) to determine the genetic variability of *C. punctatus* in the geographically important Rohilkhand plains using transferrins polymorphs as the biochemical markers and, 2) to identify the phenotypes or alleles, which might be under selection pressure due to the changing ecological conditions. For the last few decades, this region has witnessed drastic ecological changes because of deforestation and either temporary or permanent elimination of several streams and tributaries of major rivers. In addition to these changes, numerous perennial ponds and water bodies have also disappeared, which happened to be either the habitat or breeding and recruiting grounds of *C. punctatus* during monsoon months. Unless a counter mechanism is in existence, these landscape changes were likely to have noticeable effect on the genetic composition of *C. punctatus* population.

Materials and Methods

Sources and collection of fish

Between February 1998 to January 2000, a total of 978 individuals of *C. punctatus* were randomly collected from different locations of Rohilkhand plains within 77 to 80º longitude and 27 to 29º latitude (Fig. 1). The main district and cities shown in the map are : 1-Aligarh 2-Bulandshahr, 3-Khurja, 4-Moradabad, 5-Rampur, 6-Bareilly, 7-Pilibhit and 8-Badaun. The number of samples examined are shown zone-wise in table 1. From the collection sites, the fish were transported in aerated twig-knits stuffed with damp-grass to the laboratory.

Collection of Sera

Blood was drawn from live fish specimen by cardiac puncture using sterilized plastic syringe equipped with the needle # 23. To avoid hemolysis, sterile glassware and vials were preferred for transfer of blood and subsequent storage of sera. Sera from clotted blood were obtained by pipetting

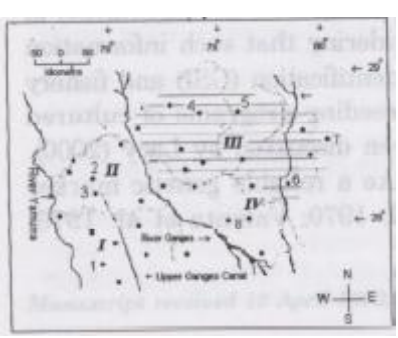

Fig. 1. Location map of Rohilkhand plains showing collection sites of *C. punctatus* samples. Main cities are shown by numbers: 1-Aligarh 2-Bulandshahr, 3-Khurja, 4-Moradabad, 5-Rampur, 6-Bareilly, 7-Pilibhit and 8-Badaun. and district boundaries by dotted lines. I-IV, subpopulations which were recognized by excess of a phenotype per pool.

out sera followed by centrifugation at 2000 rpm to clear them of contaminating blood cells. Usually the samples were analyzed fresh or within one week's storage in microfuge tubes at -20^{0}C. No changes were observed between the protein patterns of fresh and frozen sera. Protein estimation was performed according to Bradford (1976) using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis (PAGE)

To resolve proteins under native state screening of sera for Tf, phenotyping was carried out using 7.5% polyacrylamide gels in SDS-free modified protocols as described previously by Sherwani et al. (2001). Coomassie brilliant blue R-250 was used for staining followed by destaining and fixing in 7% acetic acid. Selected PAGE patterns of either type were documented on 5 or 125 ASA black and white negative films.

Identification of transferring isoforms by specific staining and western blotting

Specific staining for developing iron-binding activity of transferrins was carried out with nitroso-R. Tf nature of these bands was further verified by western blotting using pure antifish-Tf-rabbit sera raised in the laboratory in white Swiss rabbits. Essentially the protocol described by Davis et al. (1986) was followed. Human transferrin of commercial origin (Loba, India) was used as the control. Electrotransfer was made to nitrocellulose membranes (Bio-Rad, USA) and immunocross reactivity visualized following staining with 4-chloro-l-naphthol (Sigma, USA). The relative mobilities were determined and corresponded with the protein bands of collaterally run gels as well as with those developed by specific staining.

Statistical analysis

The population data was statistically treated according to known equations (Ferguson 1984).

Results

Typical PAGE patterns of sera of *C. punctatus* (978 specimens) collected from different locations (Fig.1) revealed the existence of six electrophoretic variants in β-globulin region of electropherograms (Fig. 2a). Following iron saturation of serum, these bands migrated as yellowish brown bands in PAGE and turned dark green up on nitroso-R staining (Fig. 2b). Though a few non-transferrin protein bands also took up this stain, they were faint as compared to those of Tf. More over, western blotting confirmed that each of the deep staining bands initially identified as Tfs on the basis of specific staining were actually transferrins. In an increasing order, the relative electrophoretic mobilities of isoforms A, B and C were calculated to be 0.477, 0.491 and 0.505 against a

value of 0.245 obtained for human Tf of commercial origin that was purified to homogeneity. Since codominance governs the expression of transferrin loci, six phenotypes recorded here are genotypically homozygotes AA, BB, and CC whereas AB, AC and BC are their heterozygotes with the corresponding alleles of isoforms designated as Tf*Acp*, Tf*Bcp* and Tf*Ccp*.

On the basis of proximity between the calculated phenotypic and allele frequency values of samples from different locations, four pools could be formed, which will be designated as 'subpopulations' here. Using χ^2 contingency test (Table 1), the calculated value of χ^2 was found to be 13.983, which is less than its tabulated value $\chi^2_{15,\ 0.05}$ =25. The pattern of χ^2 analysis of phenotypes also demonstrated an apparent equilibrium in the geographical distribution of the entire region. An excess of phenotype BB and BC and the deficiency of homozygote CC and the two heterozygotes AB and AC is, in any case, obvious (Table 1). Phenotype AA being extremely rare constituted only 0.5% of the total samples examined, and also in a limited area below $29_{1/2}{}^{o}$ on the western side of Ganges (Fig. 1). The areas that, on the basis of an excess or deficiency of a particular allele calculated by Students' "t" test (Table 2), show the maximum

Table 1. Phenotype frequencies of Tfcp system of *C. punctatus* in Rohilkhand plains.

District	Number of Samples Examined		Phenotypes					
			AA	BB	CC	BC	AB	AC
Subpopulation-I	100	Observed	0	30	5	43	15	7
		Expected	0.51	30.98	6.64	44.06	12.57	5.21
Subpopulation-II	650	Observed	5	210	39	280	78	38
		Expected	3.32	201.38	43.2	286.45	81.74	33.89
Subpopulation-III	150	Observed	0	43	15	67	20	5
		Expected	0.76	46.47	9.96	66.10	18.86	7.82
Subpopulation-IV	78	Observed	0	20	6	41	10	1
		Expected	0.39	24.16	5.18	34.37	9.8	4.06

Table 2. Allele frequencies of Tfcp system of *C. punctatus* in Rohilkhand plains using Student's "t" test.

	Samples Examined	Allele Frequencies		
		TfAcp	TfBcp	TfCcp
Subpopulation-I	100	0.11	0.59	0.30
Subpopulation-II	650	0.097	0.60	0.30
Subpopulation-III	150	0.08	0.58	0.34
Subpopulation-IV	78	0.07	0.58	0.35

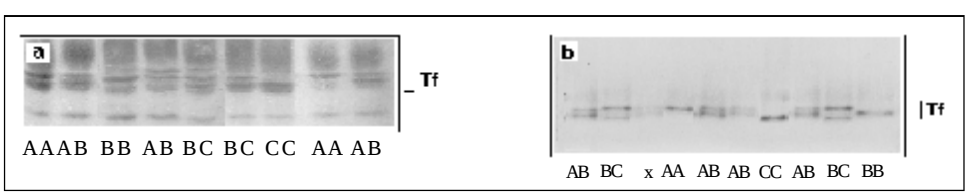

Fig. 2. Transferrin (Tf) phenotypes of *C. punctatus* as identified following protein staining (a) and specific staining (b) in native PAGE. Genotype is shown below each lane.

frequency of a specific alleles have also been identified in the same figure. On the western side of Ganges river, which is its basin of two major rivers, the Ganges and Yamuna, allele *TfAcp* had the maximum frequency between 27º and 28º latitude while that of allele *TfCcp* extended between 27º and 29º latitude. The opposite is true for allele *TfBcp,* the maximum frequency of which was observed on the eastern side of the Ganges river above $28_{1/2}$º latitude. Genetic diversity analysis suggested that the probability of nonidentity of alleles *Acp, Bcp* and *Ccp* (H_T) collected from different locations and pooled was 0.542. This result is higher than the average probability (H_S) of nonidentity, which was 0.292 for these alleles. Between the four natural pools of subpopulations, the components of gene diversity (D_{ST}) and proportion of gene diversity (G_{ST}) were found to be 0.250 and 0.462, respectively.

Discussion

There are rather few recent reports on polymorphism of transferrin in fish sera as the renewed efforts on it are mainly directed towards cloning Tf isoforms and the molecular phylogeny (Baldwin 1993; Hirono et al. 1995; Denovan-Wright 1996). Further, most of the recent and earlier works largely deal with Tfs of fish species particularly the sister species of salmon distributed in temperate regions. In these reports, genetic composition and trends of allele distribution between smaller and larger rivers, within and between hatcheries, differences in comparison with the wild populations including the changes during par-smolt transformation have been worked out (Hjort and Schreck 1982; Olin 1984; Bartley 1987; Hardiman and Gannon 1996).

In a study on GSI using polymorphism of Tf alleles, Van Doornik et al. (1996) showed that the genetic variations in Tf allele frequencies of coho salmon also differ temporally. The most extensive polymorphism happens to occur in mirror carp that has been applied to detect disappearance of certain Tf phenotypes from the gene pool (Valenta et al. 1976, Csizmadia et al. 1995). The *Barbus* species was found to be polymorphic which has distribution in tropical as well as temperate waters (Stratil et al. 1983). An alphabetic nomenclature (A, B, C to H) for Tf isoforms in increasing order of relative electrophoretic mobilities have been designated in the literature and the same system has been followed for three isoforms of *C. punctatus* on which some preliminary observations have been published (Hasnain et al. 1981).

On the basis of frequency of Tf alleles in fish, the highest value of the mean heterozygosity (H_{mean}) has been recorded to be 0.425 (Van Doornick et al. 1996). H_{mean} of natural population of *C. punctatus* distributed in the Rohilkhand plains is 0.363 that reflected a higher magnitude of polymorphism. The proportion of gene diversity G_{ST} (0.462) and the component of gene diversity D_{ST} (0.250) demonstrated considerable variability among the samples of different subpopulations which means there is intermixing of populations. The grouping into subpopulations was decided on the basis of closely similar values of allele frequencies in a specific area designated as zones I-IV. This indicated that the distribution of *C. punctatus* subpopulations is determined by natural

factors such as the terrain rather than the arbitrary district boundaries (shown by dotted line in figure 1). Since the subpopulations were also panmictic and phenotypically in equilibrium, there have to be factors or an apparent mechanism that might have countered the probable negative impact of ecological changes. The above observation may be the consequence of the combined effects of two major factors: 1) monsoon flooding of low lying areas of the plains, which still permits some intermixing, 2) the positive effect of human activity, that is, the frequent transportation of catches across the district boundaries and, 3) a contribution of nocturnal terrestrial migration as an air-breather. The last factor may be the least contributing since *C. punctatus* is a poor air-breather and the migration should therefore be restricted to only closely located water bodies. In any case, one phenotype AA and its corresponding allele *TfAcp* appear to be under selection pressure. This is evident by statistical analyses including the mean, range and standard deviation of both the homozygotes and heterozygotes (Fig. 3). Since all possible combinations of Tf isoforms including homozygous condition AA occur in the natural population of *C. punctatus*, alleles were not lethal. Homozygous population BB, on the contrary, appear to be at an advantage compared to its heterozygotes, therefore, this fish is an exception because the homozygous progeny has a survival rate compatible to that of heterozygotes.

The relative abundance of various phenotypes in fish populations depends on several factors such as the differences in fertility and post-hatching viability of the progeny (Hershberger 1970). The last factor may, in turn, be related with the changes in ecological conditions and inherent susceptibility to pathological conditions. Collectively, each of the above factors constitutes the selection pressure that has already been recorded in certain specific cases. For instance, Suzumoto et al. (1977) reported that in coho salmon, a Tf allele could serve as the marker of resistance to bacterial kidney disease, while the studies of Winter et al. (1980) limited the finding to particular stocks only. Available data also shows that among freshwater fishes, species belonging to genus *Channa* can be placed in the group of disease-prone species (Nabi et al. 2000).

In the case of *C. punctatus* populations investigated here, remarkable differences in the fertility of BB or CC might not exist because the heterozygote BC is quite in abundance. It is more probable that the observed variations in the relative abundance of AA are due to diminished post-hatching viability of these homozygotes where changes in ecological condition and inherent susceptibility might have had some role. The alternate or the additional factor may

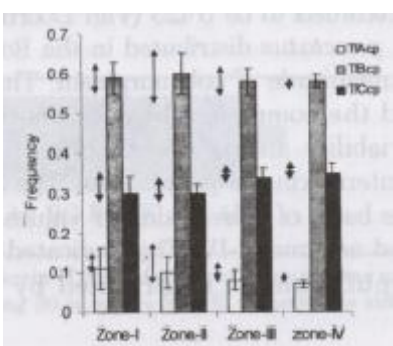

Fig. 3. Frequency distribution of fish transferring alleles TfAcp, TfBcp and TfCcp showing mean, range (↔) and standard deviation (I) in different zones as recognized on either side of Ganges river following statistical analysis. Details under Results and Discussion.

be a relatively low iron-binding capacity of this phenotype that would be further enhanced if low affinity Tf receptors exist on the cell surface, since intake of Tf-bound iron at the site of unloading is receptor-mediated endocytosis (Lim and Morgan 1984). In a codominant system, the functional deficiency is compensated in heterozygous condition, that is, in AB or AC, which is positively reflected in statistical analysis of phenotype or allele frequencies.

Though within subpopulations identified here, the gene flow appears to be unrestricted. *C. punctatus* subpopulations in Rohilkhand plains are in the future, likely to be under more detectable duress generated by the environmental and ecological changes that are taking place in this region. To prevent a shift in equilibrium and to keep them panmictic, future efforts in their management have to be focused on avoiding further destruction of their seasonal breeding grounds and habitats, preserving monsoon month connectivity of such locations and controlling illegitimate fishing during these months.

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